USE OF FLUORESCENT DYES AND SPECTROFLUOROMETRY TO OBSERVE EVIDENCE OF VESICANT TOXICITY IN HUMAN EPIDERMAL CELLS

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ABSTRACT

Normal human epidermal keratinocyes (NHEK) show multiple dose-related biochemical changes at 3 hours after in vitro exposure to a vesicant compound, 2-chloroethyl ethyl sulfide (CEES). CEES in ethanol was diluted to 0.8, 8.0 and 80 mM concentrations in cell culture medium over confluent NHEK on gel-coated membranes of Millipore Millicells® or in NHEK suspensions. A site-specific fluorescent dye was incubated with each NHEK layer for 1 hr prior to comparisons of normal and challenged NHEK within a Cytofluor® 2300 spectrofluorometer. Reduced fluorescence from loss of all dye probes indicated severe membrane damage with 80 mM CEES in medium. Intracellular increases in Ca^{++}, evidence of altered mitochondrial activity, and decreases in pH, glutathione levels and lysosomal integrity, were observed with 0.8 and 8.0 mM CEES in the culture medium. Control studies performed with Testskin® and another human epidermal model suggest that dermal substitutes and transportation stresses can influence results with the dye probe/Cytofluor 2300 method. However, the feasibility of using the described methods to observe vesicant biochemical effects, screen antivesicants and perform other toxicological studies with NHEK models is supported by the results of the preliminary studies.

INTRODUCTION

Vesicants have been used in past wars and remain as threats. Therefore, antivesicant materials are needed to protect personnel. Prior to use, antivesicants must be judged as safe and effective during vesicant challenges of suitable models. Such models must reasonably represent effects of vesicants in man so that measurements of effectiveness can be extrapolated to man.

The hairless guinea pig has been used as a test model[1,2] for the erythema, edema and vesication that may occur within 24 hours after liquid or vapor exposures of human skin to HD[3]. In the skin of guinea pigs[1,2] or other animals[4,5] after HD exposures, the observed erythema and edema are not visibly different from animal responses to other causes of inflammation. However, gross vesication in animals is relatively rare[3,4]. The structural, functional and biochemical differences of animal and human skin limit some histological comparisons[4], but histopathology uniformly shows cytotoxic effects of HD on epidermal basal cells[1,7]. Grafts of excised human skin to animals have been tried[5], but uniformity of grafted test specimens has been difficult to achieve. The hairless guinea pig model displays gross and microscopic effects of HD that are uniform, graded and measureable[2]. However, use of animal models could be avoided if alternative models can be developed.

A search for alternative models was begun with the concept that artificial human skin might substitute for animals and man. Living Skin Equivalent® (LSE®, also called Testskin®) was provided as a gift of Organogenesis, Incorporated (Cambridge, MA). According to the vendor, LSE fabrication from cultured human fibroblasts and collagen (Living Dermal

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Equivalent^{R}, LDE^{R}) was by apical seeding of the LDE with normal human epidermal keratinocytes (NHEK) obtained from surgical specimens; after differentiation, the uppermost layer of NHEK was lifted above liquid nutrient medium to form a cornified surface in air. Specimens of LSE, challenged with HD vapor cups[2], were found to have gross and histological signs of mustard damage[6] that resembled signs reported in skin of men exposed to HD[5,7]. Therefore, Testskin became a potential model for use in screening of candidate antivesicant compounds.

Diverse fluorescent dyes have been used to provide multiple end points[9] in a system for observing cytotoxicity[9,10] in cultured human cells[11]. The major components[8] of this system are (1) cultured NHEK, (2) intracellular dye probes, and (3) the Cytofluor 2300 (Millipore Corporation, Bedford, MA). This instrument is an automated spectrofluorometer that directs ultraviolet light upward through cell culture plates[8]. Some dyes fluoresce inside of the illuminated cells and emit signals that are measurable by the instrument[11].

Fluorescent probes are identified below by names or acronymns used by the vendor (Molecular Probes, Eugene, OR). Chemical names and references for the following information are available in the Molecular Probes Handbook and elsewhere[8,12]. The acetoxymethyl ester (AM) confers membrane permeability to certain probes and they have been designated with the -AM suffix. Once inside cells, the AM is cleaved to make the probe less permeable[8,12]. For example, non-fluorescent calcein-AM is cleaved to make fluorescent calcein, which is well retained by normal cell membranes[12]. Reduced calcein levels indicate degrees of plasma membrane damage. Therefore, a calcein control is used to monitor possible leakage of other probes. Some cleaved dyes fluoresce after binding Ca^{++} (fluo3) or glutathione (CMFDA). Other dyes indicate intracellular pH (BCECF-AM) or localize in cellular compartments such as lysomes (neutral red) or mitochrondria (rhodamine 123).

EXPERIMENTAL OBJECTIVE

The objective of this project was to determine the feasibility of concurrently using human skin cells/models, fluorescent probes, and the Cytofluor 2300 instrument to measure toxic effects induced by vesicant compounds, antivesicant compounds or both together.

APPROACHES

1. Use of multiple end point methods to explore variables affecting dye retention of fabricated human epidermal models.

2. Exposures of epidermal models to dilutions of 2-chloroethyl ethyl sulfide, followed by incubation with diluted dye probes and Cytofluor 2300 measurements.

MATERIALS AND METHODS

At the onset of this study it was expected that epidermal models would be delivered to Aberdeen Proving Ground, MD for challenge with sulfur mustard (HD), as had been done with Testskin[5]. It was not known if the HDE substrate of Testskin would sequester probes, thereby resulting in a high background signal. Control testing with dyes was planned but Testskin was not immediately available. Therefore, initial control tests were performed with a non-commercial human epidermal model that had been used in a multiple end point assay[11]. A modification of this model (as described below) was used to see if it could be prepared in Binghamton, NY, for use in MD. Unfavorable results (reported below) led to the decision to conduct control studies and CEES challenges in NY. Work with NHEK monolayers and confirmatory studies with CEES were conducted later at APG.

A. HUMAN SKIN SUBSTITUTES FOR CONTROL STUDIES

Preliminary control testing involved use of a proprietary human epidermal model (HEM). Millipore Corporation graciously granted use of their patented technique for overlaying Millicell CM^R microporous membranes with "gel-coating" to make "gel-membrane" and provided inserts for use according to the methods described by Cock et al[11]. This method was abridged, as described below.

Second passage cell strains of NHEK and 0.025% w/v trypsin/0.01% w/v EDTA solution (CC5013) were obtained from Clonetics Corporation (San Diego, CA). NHEK were maintained in serum-free keratinocyte growth medium (KGM) during propagation on the plastic surfaces of cell culture (T) flasks. NHEK dissociated with CC5013 were suspended in KGM containing 10% fetal calf serum/1.5 mM calcium chloride, and seeded onto gel-membranes at a density of 3-6 x 10^5 cells/cm^2. Cells were allowed to stratify for 5 to 7 days. The medium was changed daily while the stratifying NHEK were maintained at 37°C, in 5% carbon dioxide/air. In this effort, HEM preparations were used before they matured. To achieve mature HEM by the methods of Cook et al[11], the apical medium was removed from the inside of the insert, exposing the apical surface of the stratified NHEK to carbon dioxide/air for additional days (5 to 7) of stratum corneum formation.

While Testskin was unavailable, the specimens of immature HEM (3-4 cells thick on gel-membrane) were transported to Aberdeen Proving Ground, MD from Binghamton, NY, under three different sets of conditions. Effects of transportation were checked with two dyes. When available, Testskin was used intact, or without epidermis. Eight fluorescent probes were tested as candidates for use in the available epidermal models.

B. FLUORESCENT PROBE ASSAYS OF HEM AND TESTSKIN

Eight different assays were accomplished with Testskin and HEM tissues. Seven assays had protocols nearly identical to those described in detail by Cook et al[11]. Briefly, Testskin was washed three times in DMEM (Dulbecco's Modified Eagle's Medium without phenol red; GIBCO-BRL, Grand Island, NY) at 37°C. Similar washing was performed with HEM or gel-membrane (dermal equivalent).

Each skin model was incubated for one hour with one of seven fluorescent dye probes or exposed to sodium fluorescein. The dyes and concentrations used were the following: calcein-AM (10 ug/ml), CMFDA (10 ug/ml), neutral red (100 ug/ml), BCECF-AM (50 ug/ml), fluo3-AM (25 uM), rhodamine 123 (10 ug/ml) or 2',7'-dichlorofluorescein diacetate (2,7 DCF; 100 uM). After incubation, each preparation was washed three more times with DMEM and analyzed in the Cytofluor 2300, using the filter sets and gain levels listed in Table 2.

To determine dye uptake of epidermal substrates, fluorescence readings from the full-thickness models were compared with readings from substrates without epidermis. Signals from LSE were compared with those from the LDE that remained after epidermis was stripped off with a fine forceps (subsequent to dye loading of the LSE). Signals from HEM were compared with those from gel-membrane preparation.

Sodium fluorescein was used to assess epidermal permeability by using a different protocol from the one used with the seven fluorescent probe assays listed above. Briefly, after washing the LSE or HEM three times in DMEM, 200 ul of a 10 ug/ml sodium fluorescein (Sigma Chemical Co., St. Louis, MO) solution was placed on the Testskin in an apical chamber. The apical chamber was made by using the silicone sealant, assay rings and directions provided with Testskin, thereby creating a well to hold dye away from the medium

under the Testskin. After 120 minutes (30 minutes for the HEM) the medium in the basal compartment was assayed within the Cytofluor 2300 using the filter sets and gain levels listed in Table 2. The amount of sodium fluorescein in the medium (basal compartment), relative to the controls, was considered to be a measure of epidermal permeability.

C. TESTING OF CONFLUENT NHEK ON GEL MEMBRANES

A model to simulate the cellular layer located immediately above the basal membrane of human skin was prepared by adapting the methods of Cook et al[11]. Some Millicell CM inserts were gel-coated, with permission granted before Millipore Corp. licensed their coating and HEM technology to MatTek Corporation (Ashland, MA). Later studies involved use of NHEK grown on inserts coated by MatTek Corp. The gel coating was applied free of charge by MatTek Corp. under a cooperative research and development agreement (CRDA).

CEES 8.56 M liquid (Aldrich Chemical Co., Milwaukee, WI) was serially diluted in anhydrous ethanol to make working solutions of 4.28 M, 42.8 mM and 428 mM fresh each day. Neat ethanol was used in control preparations. A 10 ul aliquot of a CEES dilution, or ethanol, was added to 490 ul of DMEM in the apical compartment of each insert. To prevent hydrolysis of CEES before NHEK exposure, the aliquot and medium were mixed immediately in the 1 ml disposable tip of a Pipetman® (Rainin Instrument Co., Woburn, MA). NHEK on inserts were held for one hour with CEES under ambient conditions, washed twice with DMEM and incubated at 37°C. Neutral red and sodium fluorescein dyes were dissolved in DMEM. The six other dyes were dissolved in dry DMSO (Sigma) before addition to DMEM. NHEK were incubated in 10, 50 or 100 ug/ml solutions of dye in DMEM at 37°C for one hour. The Cytofluor 2300 was used four hours after the onset of CEES challenges.

NHEK suspensions were used in some experiments to prevent any problems with dissociation of cells from the plates during the chelation of calcium. Suspensions were prepared after multiplying cells in T-flasks. Published methods[11] were used, except that harvesting was done as follows: cells at 50% confluency were washed briefly with 0.1% trypsin in physiologic buffered saline solution with 5 mM EGTA (ethylene bis(oxyethylenenitrilo)-tetraacetic acid, Aldrich Chemical Co., Milwaukee, WI). This fluid was replaced for 1 min and decanted again. The T-flasks were then capped and held at room temperature until 10% of the cells lifted from the plastic when tapped from below. Trypsin neutralizing solution (Clonetics) was then added as per manufacturer's directions.

To avoid variables of dye loading and cell counts, cells from one flask were incubated with calcein-AM; cells from another flask were incubated with CMFDA. Cells in each flask were loaded with dye for one hour at 37°C before they were split into two sets of 4 tubes (8 total). NHEK suspension mixtures were made by adding 120 ul of a CEES dilution, or ethanol, to 5880 ul of the suspension. The tubes were promptly capped and inverted twice to accomplish mixing. Cells in each tube were incubated (with or without calcium) for 3 hours after CEES was added, and centrifuged for 10 min before the supernatant was decanted. The NHEK in each tube were resuspended, divided into equal volumes in 3 wells of a 24- well plate and assayed in the Cytofluor 2300.

RESULTS AND DISCUSSION

A. EFFECTS OF TRANSPORTATION OF IMMATURE HEM

HEM were assayed for damage to epidermal cells, in NY before transportation, after 5 hrs in transit (to APG, MD) followed by 18 hrs of incubation, or after 5 hrs in transit during

a total time of 24 hrs without incubation. Data given in Table 1 suggest that the non-viable control (gel-membrane) may retain slightly more calcein (100 unit changes) as time enroute increases. However, the viable HEM appear to leak calcein after transport (>2000 unit change). The sodium fluorescein assay suggests that gel-membrane offers a slight barrier to dye leakage, but this barrier is compromised after 24 hrs enroute. Normal resistance of HEM tissue to intercellular dye movement appears to be lost rapidly with increased travel time. One explanation of these observations is that increasing transportation stress may induce HEM cells to separate while plasma membrane damage remains minimal.

TABLE 1

TRANSPORTATION EFFECTS ON FLUORESCENT DYE READINGS

Barrier to Dye	Dye	Control	5 hr Trip	24 hr trip
Gel-membrane	calcein-AM	100*	200*	300*
HEM Plasma membrane	calcein-AM	4200*	2000*	1600*
Gel-Membrane (leakage through)	sodium fluorescein	4300*	4300*	6300*
HEM cells (leakage between)	sodium fluorescein	500*	2200*	6450*

* Cytofluor 2300 fluorescence units (values rounded off). n=3.

B. OBSERVATIONS ON FLUORESCENT DYE DISTRIBUTIONS

Testskin was incubated with eight dyes of differing site specificities to see if fluorescent dye binding to dermal tissue would hinder attempts to read fluorescence changes in epidermis. Table 2 shows most of the fluorescent signals from the first six dyes as coming from LSE epidermis. Similar comparisons with HEM and gel-membranes also showed most of the signal in HEM epidermis (Van Buskirk, unpublished data)

Table 2 shows different results with 2,7 DCF (free radicals) and sodium fluorescein (confluence of cells) than with the other dyes. Differences between test conditions appear to account for these differences. Dermal cells are likely to be damaged during use of forceps to strip and discard the epidermal portion of LSE. The current hypothesis is that extra dye is bound by free radicals generated in damaged dermal cells, thereby accounting for greater fluorescence in the dermal substrates than in the whole LSE (5000 vs 1693). It remains to be seen if HD generates free radicals detectable with 2,7 DCF. The values obtained with sodium fluorescein diverge because the signal is from dye that passes between the cells, not through their plasma membranes[8]. Table 2 shows LSE as an excellent barrier to sodium fluorescein (A,C) until epidermis is removed (B).

Tables 1 and 2 suggest that (1) the probe/Cytofluor method produces readable signals with both testskin and immature HEM, (2) additional testing will be required to exclude any of the eight original probes from use in screening tests, (3) measurements of epidermal signals are not precluded by binding of probes to dermal equivalents, and (4) non-cornified HEM specimens may have suffered membrane damage and losses of intercellular adhesion during transportation.

TABLE 2

FLUORESCENT PROBE/CYTOFLUOR 2300 ASSAYS OF LSE* AND LDE**

Parameter	Dye	A LSE +dye	B LDE +dye	C LSE only	D Net signal	E S/N ratio	F Filter set	G Gain
Plasma membrane integrity	calcein-AM	3004 ±294	1303 ±196	521 ±66	1701	3.18	485/530	4
Glutathione	CMFDA	8804 ±293	1475 ±60	103 ±13	7329	6.34	485/530	3
Lysosomes	neutral red	6540 ±186	648 ±55	116 ±12	5892	12.07	508/645	4
Intra-cellular pH	BCECF-AM	3575 ±270	1346 ±156	521 ±66	2229	3.7	485/530	4
Intra-cellular Ca++	fluo3-AM	4685 ±327	2091 ±161	1875 ± 15	2594	13.01	500/550	5
Activity of mitochondria	rhodamine 123	5732 ±252	1494 ±228	16 ±2	2594	3.87	500/550	2
Free radicals	2,7 dichloro-fluorescein	1693 ±200	5000 ±534	103 ±13	-3307 (see text, above)	0.32	485/530	3
Epidermal permeabil.	sodium fluorescein	15 ±0	8383 ±517	14 ±0	8368 (see text, above, & #, below)	8369	485/530	3

* LSE (Living Skin Equivalent) and LDE (Living Dermal Equivalent) are registered trademarks of Organogenesis, Inc, Cambridge, MA.
** In this case, LSE with epidermis removed to leave LDE.
± Standard error of the mean.
A Includes autofluorescence from epidermis and LDE. (#: leakage of dye between cells)
B Omits epidermal autofluorescence. (#: measures leakage through LDE)
C Omits dye fluorescence. (#: signals autofluorescence of media)
D Net epidermal autofluorescence, A-B. (#: A-B= sodium fluorescein signal)
E Signal/noise ratio, A-C/B-C.
F Filter sets used in the Cytofluor 2300 with given dye.
G CytoFluor 2300 sensitivity level (gain) used for given dye.

C. EFFECTS OF CEES IN CONFLUENT NHEK ON GEL-MEMBRANES.

Selection of the three CEES concentrations listed in Table 3 was based on very limited data from a dosing experiment which identified a minimally observable response in another test system[13]. We used this data to select a conveniently deliverable concentration (8.0 mM); this was increased and decreased by one log for evaluation of the probe/Cytofluor system.

An ephemeral milky trace was observed during addition of 8.0 mM CEES (in ethanol) to DMEM[14]. A transient milky plume appeared with additions of 80 mM concentrations[14,15]. These observations suggest that CEES separated from the DMEM and concentrations in the medium less than expected. Also a few pits, nearly microscopic, were subsequently observed in the underlying polystyrene of the culture plates which were used. This result suggests that droplets of liquid CEES were delivered onto the confluent NHEK and dissolved the underlying plastic.

The data in Table 3 suggest that 0.8 and 8.0 mM CEES causes little calcein dye leakage, after 3 hours, from NHEK on inserts or in suspension. The leakage of calcein in both cases and CMFDA from NHEK in suspensions appears to be sharply increased with 80 mM CEES. Other dye probes show different effects in NHEK on inserts: readings for intracellular fluo3 (Ca^{++}) and rhodamine 123 (mitochondrial activity) increase with 0.8 and 8.0 mM CEES, respectively. CMFDA (glutathione) readings increase with 0.8 mM CEES and decrease with 8.0 mM CEES. Results with BCECF (pH) and neutral red (lysosomal integrity) suggest that these dyes, and possibly 2,7-DCF (free radicals), may reveal effects of 0.8 mM CEES. These results suggest that several dyes may be applicable to antivesicant screening if concentrations of vesicant are kept below levels causing marked membrane damage or vesicant precipitation.

The dose-related increase of fluorescence readings (Table 3, top) associated with intracellular Ca^{++} (fluo3) and a decrease related to lysosomal lysis (neutral red) led to the hypothesis that CEES could act as an ionophore to pass Ca^{++} into cells and cause damage by inducing proteases. This hypothesis was tested by comparing results with calcein, CMFDA or fluo3 in NHEK, with and without chelation of Ca^{++} by EGTA in the medium (Table 3, bottom). Although fluorescence levels differed from those with inserts, suspension test results showed respective concentrations of CEES to have similar effects on calcein, CMFDA, and fluo3 readings, with or without chelation. Therefore, the hypothesis and CMFDA results with inserts (Table 3, top) were not confirmed by suspension studies. However, the possibility of Ca^{++} release from internal stores, with concurrent leakage of Ca^{++} through the cell membrane, is not excluded.

Additional experiments were performed at APG to confirm the original results obtained in Binghamton. Several studies using calcein and fluo3 (data not reported) revealed variations in fluorescence reading levels, while confirming the patterns reported above. The dose-related increase of Ca^{++} and the depletion of glutathione (CMFDA, Table 3) were confirmed by these later studies. Other data from these studies are given in Table 3, with interpretations as follows. Alterations in mitochondrial activity (rhodamine 123) were observed. Parallel studies revealed little or no free radical activity, whether the dye was loaded before or after CEES challenges. An observed gradual decline of BCECF (pH) readings with increasing CEES concentrations was confirmed.

TABLE 3

CYTOFLUOR 2300 READINGS AT THREE HOURS AFTER ONE HOUR EXPOSURES OF NHEK AS CONTROLS AND WITH THREE CONCENTRATIONS OF CEES[a]

CELLULAR FUNCTION	DYE	0 mM	0.8 mM	8.0 mM	80 mM
	Results with NHEK on Millicell CM inserts.				
Plasma membrane integrity	Calcein-AM (10 ug/ml)	4007 (4)[b]	3812 (104)	3660 (873)	1003 (175)
Glutathione	CMFDA (10 ug/ml)	2185 (65)	2462 (92)	1496 (72)	1249 (189)
Intracellular Ca^{++}	Fluo3 (10 ug/ml)	2748 (102)	3665 (98)	6634 (40)	5786 (56)
Mitochondrial activity[c]	Rhodamine 123 (10 ug/ml)	2278 (375)	2809 (743)	5073 (661)	3334 (597)
Intracellular pH[c]	BCECF-AM (50 ug/ml)	1356 (43)	1250 (3)	1119 (86)	888 (145)
Lysosomal integ.	Neutral Red (100 ug/ml)	3287 (260)	2449 (93)	1940 (19)	1968 (28)
Free Radicals (dye after CEES)	2,7-DCF (100 ug/ml)	7494 (869)	8217 (332)	7027 (804)	4978 (124)
	Results with suspensions of NHEK.				
Plasma membr. integ. (with 1+ mM Ca^{++})	Calcein-AM	8371 (151)	8153 (92)	7706 (32)	(3045) (13)
Plasma membr. integ. (with 5 mM EGTA)	Calcein-AM (57)	8392 (71)	8681 (20)	7685 (10)	(1901)
Glutathione levels (with 1+ mM Ca^{++})	CMFDA	4078 (145)	4051 (111)	3941 (63)	1577 (21)
Glutathione levels (with 5 mM EGTA)	CMFDA	3659 (198)	3811 (94)	3704 (36)	1556 (27)
Intracellular Ca^{++} (with 5 mM EGTA)[d]	Fluo3	1117 (3)	1200 (3)	1242 (4)	1148 (38)

[a] 2-chloroethyl ethyl sulfide.
[b] Parentheses indicate standard error of the mean (for n=3).
[c] Data from studies performed at APG, MD.
[d] Data obtained separately from other suspension experiments.

Data in Table 3 suggest that (1) results with CEES justify progression to studies with sulfur mustard, (2) a relatively lower range of challenge concentrations should be used, (3) dye probe/Cytofluor 2300 examinations should be made at several post-exposure intervals, and (4) diverse and multiple end point responses can be obtained with available probes.

CONCLUSIONS

1. Human epidermal keratinocyte models are capable of displaying multiple end point responses to a vesicant compound following post-exposure incubation with fluorescent dye probes and analysis in a Cytofluor 2300 spectrofluorometer.

2. The described methods may be useful for observations of sulfur mustard's toxic effects, the screening of compounds for antivesicant activities and to obtain other toxicological data.

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